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Determination of Plasma Lipid Hydroperoxides by an NADPH/NADP$^+$ Coupled Enzyme Reaction System. Evaluation of a Method

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Summary: Several techniques based on different principles have been proposed to measure lipid hydroperoxides. Enzymatic methods are sensitive and can be quite specific but they are subject to interference by inhibitors and not all are stoichiometric. The present work proposes some modifications of the *Heath & Tappel* (Anal Biochem 1976; 7:184–91) enzymatic method of determination of lipid hydroperoxides in order to standardize and automate it and to meet the analytical criteria required for a biological assay. The proposed new protocol and the automated assay give acceptable within-run and between-run precisions, with coefficients of variation of 3.34% and 5.80%, respectively, at the usual plasma lipid hydroperoxides content. The recovery is 98.7 ± 4.89%, and the method is linear for a wide range of contents and sensitive (10 μmol/l) enough to measure the plasma lipid hydroperoxides content.

Introduction

Oxidative stress is involved in the physiopathology of several diseases (1). Nonetheless, the degree of oxidative stress is not usually measured in clinical practice, in part because there is no unanimously accepted method for doing so (2, 3).

A number of techniques has been developed to detect lipid peroxidation in plasma. A first group included the thiobarbituric acid test, diene conjugation, detection of malondialdehyde, other aldehydes and hydrocarbons. None of these methods are specific for hydroperoxides, and all of them are subject to false interpretations because of interference by non-peroxidized compounds (4).

The second, more specific group of techniques, is based on the determination of lipid hydroperoxides (5, 6). Lipid hydroperoxides are relatively stable products, resulting from a free radical attack on lipids, and therefore measuring it can supply us with information about the degree of lipid peroxidation.

Several techniques based on different principles have been proposed to measure lipid hydroperoxides. The reference method is based on the iodide oxidation by lipid hydroperoxides, but its sensitivity is low when oxygen is present; it is useful only in the case of pure lipid solutions (7). Colorimetric techniques based on phenolphthalein or methylene blue oxidation have also been used (8, 9), as have fluorometrical ones like oxidation of dichlorofluorescein by hydroperoxides in the presence of haematin (10) or the oxidation of homovanillic acid to a fluorescent dimer (11).

The following enzymatic methods have also been developed:
Stimulation of cyclooxigenase-synthetase activity by hydroperoxides. The enzymatic activity determined by measuring the oxygen consumption is proportional to the lipid hydroperoxides concentration (12).

Lipid hydroperoxides are reduced by glutathione through the action of glutathione peroxidase[1]. The enzymatic reduction is followed by fluorometry (13):
The enzymatic method described by *Heath & Tappel* (14) involves a coupled glutathione peroxidase-glutathione reductase[1] reaction: the enzyme glutathione peroxidase catalyses the reduction of lipid hydroperoxides in the presence of glutathione, a timed reaction of glutathione peroxidase coupled with the oxidation of NADPH by glutathione reductase that allows a direct spectrophotometric measurement of hydroperoxides. This method is most usually applied to determine lipid hydroperoxides in plasma, although it is not free of problems (15, 16). Its intra- and inter-assay precision is good, but its reaction kinetics have been found to differ in the case of samples and standards, and this leads to a lack of specificity.

The present work proposes some modifications of the *Heath & Tappel* method in order to standardize and automate it and fulfill the analytical criteria required for a biological assay.

[1]) Enzymes:
Glutathione peroxidase (EC 1.11.1.9)
Glutathione reductase (EC 1.6.4.2)

Materials and Methods

Reagents

The reagents needed for the assay were buffer 124 mmol/l Tris-HCl (pH 7.6)/0.2 mmol/l EDTA (Panreac); reduced glutathione, 4.25 mmol/l; NADPH, 2 mmol/l; glutathione reductase (EC 1.6.4.2 type II from baker's yeast), 100 kU/l; glutathione peroxidase (EC 1.11.1.9 from bovine erythrocytes), 16 kU/l; bovine serum albumin (fraction IV), 65 g/l containing 9 g/l NaCl; and butylated hydroxytoluene 20 g/l in absolute ethanol. All were purchased from Sigma Chemical Co. (St. Louis, MO 63178, USA).

Standard *t*-butyl hydroperoxide: A stock solution was prepared by diluting 70% *t*-butyl hydroperoxide (Sigma Chemical Co.) with absolute ethanol to 60 mmol/l.

Manual procedure

In the *Heath & Tappel* assay 700 µl of buffer 124 mmol/l Tris-HCl (pH 7.6)/0.2 mmol/l EDTA and then 200 µl of the sample (blank, standard, plasma) were introduced into a 1 ml microcuvette and incubated at room temperature for 5 min. Fifty µl of NADPH (2 mmol/l), 10 µl of glutathione peroxidase (16 kU/l) and 100 µl of reduced glutathione (4.25 mmol/l) were successively added. The whole was incubated at 33 °C for 15 min and the spectrophotometric absorbance at 340 nm was measured. After addition of 10 µl of glutathione reductase (100 kU/l) and incubation at 33 °C for 15 min a second spectrophotometric measurement at 340 nm was carried out to determine the amount of oxidized NADPH, and the lipid hydroperoxides content was calculated. The difference between the two absorbances is proportional to the sample hydroperoxide content, and the values are obtained by comparison with standards of *t*-butyl hydroperoxide (25 to 300 µmol/l) prepared by an aqueous dilution of the 60 mmol/l stock solution.

The modifications of the method proposed are:

- Lipid peroxidation was stopped by adding 10 µl of the 20 g/l butylated hydroxytoluene solution to each ml of sample (plasma, blank and standard).
- All incubations were carried out at room temperature and the incubation times were modified as follows: 5 min after the addition of glutathione peroxidase and 10 min after the addition of glutathione reductase.
- To obtain the calibration curve, *t*-butyl hydroperoxide in a solution of 65 g/l bovine albumin containing 9 g/l NaCl treated in the same manner as the assay samples was used.

The calibration curve was plotted as follows: the abscisa indicates the contents of *t*-butyl hydroperoxide as a function of the difference between the two absorbances.

Automated assay

Automated assay was performed using a Cobas FARA centrifugal analyzer (Roche Diagnostics, Nutley, NJ 07110, USA). All reagent additions, mixing, spectrophotometric measurements and calculations were carried out automatically according to the programmed instructions.

The automated assay of lipid hydroperoxides was carried out by using the same proportion of reagents and sample as in the manual procedure. The Tris HCl/EDTA buffer was added to the assay reagent container and the NADPH, reduced glutathione and glutathione peroxidase mixture was added to the starting reagent container. Assay reagent (175 µl) and sample (50 µl, with 50 µl rinse) were added to cuvettes, mixed and incubated at room temperature for 5 min. Then 40 µl of starting reagent were added, mixed in the cuvette and let stand for 5 min. During this time the starting reagent container was manually exchanged for the one containing glutathione reductase solution. After the 5 min incubation the absorbance was measured at 340 nm, and then 4 µl of glutathione reductase solution were added to the sample probe and mixed. Ten min later the spectrophotometric absorbance at 340 nm was measured. A series of standards was analyzed in each assay session, and the concentration of the standards, including the blank, was introduced into the program instructions and the values of the samples were obtained by comparison with *t*-butyl hydroperoxide standard.

Interferences

The potential matrix interference was tested by comparison, covariance analysis, of the slopes of the calibration curves obtained with and without addition of the plasma pool.

Results

Comparison of the kinetics of NADPH disappearance during the incubation steps of a standard and a plasma sample (fig. 1) leads to the following conclusions:

In the *Heath & Tappel* assay the absorbance of the plasma reaction mixture continues to decrease during the incubation time prior to the addition of glutathione peroxidase to the assay tube. Similarly, after the addition of glutathione reductase, a regular decrease in the absorbance was observed well beyond 15 min, unlike what happened with the absorbance of the standard solutions that rapidily achieved stabilization, both before and after the addition of glutathione reductase. We considered the effect observed in samples to be due to the lipid peroxidation process.

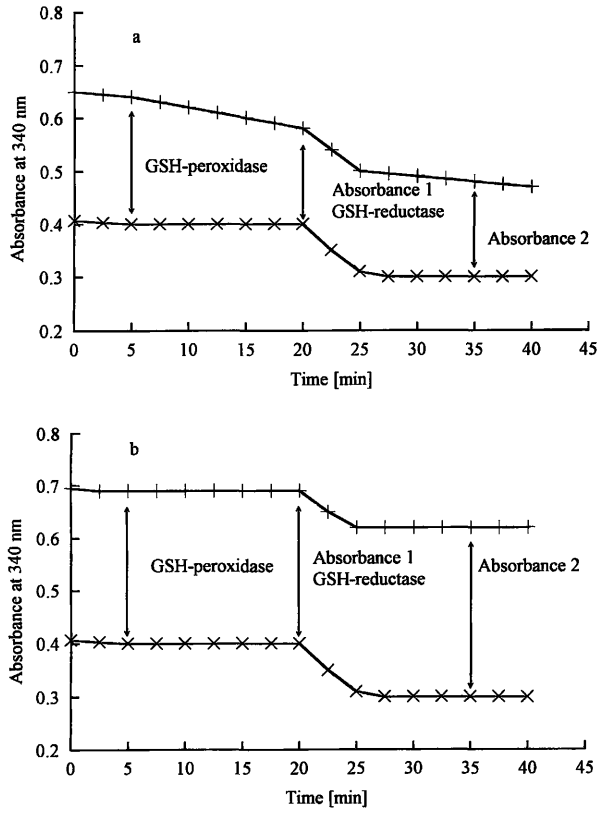

Fig. 1 Plasma lipid hydroperoxides determination. Kinetics of NADPH disappearance during the incubation steps of a standard of 100 mmol/l (x) and a plasma sample (+) without (a) and with (b) butylated hydroxytoluene addition. Absorbance 1 (absorbance value prior to the glutathione reductase addition); and Absorbance 2, (absorbance value at 15 min after glutathione reductase addition).

When butylated hydroxytoluene was added and the samples were incubated at room temperature, the absorbance during the incubation period prior to glutathione reductase addition was stable after a few minutes, and after the addition of glutathione reductase the fall in absorbance was very evident from the first minutes. The end of the reaction was more complete and rapid; only 10 minutes were needed (fig. 1).

According to these results the assay was modified by adding 10 µl of the 20 g/l butylated hydroxytoluene solution to each ml of sample (plasma, blank and standard) to stop the lipid peroxidation process and all incubations were carried out at room temperature. The incubation times after addition of glutathione peroxidase and glutathione reductase were 5 and 10 min, respectively.

Matrix interferences

Two sets of standards were prepared:

- standard solutions of *t*-butyl hydroperoxide, 25 to 300 µmol/l and
- standard solutions of *t*-butyl hydroperoxide, 25 to 300 µmol/l added to a constant amount of plasma pool.

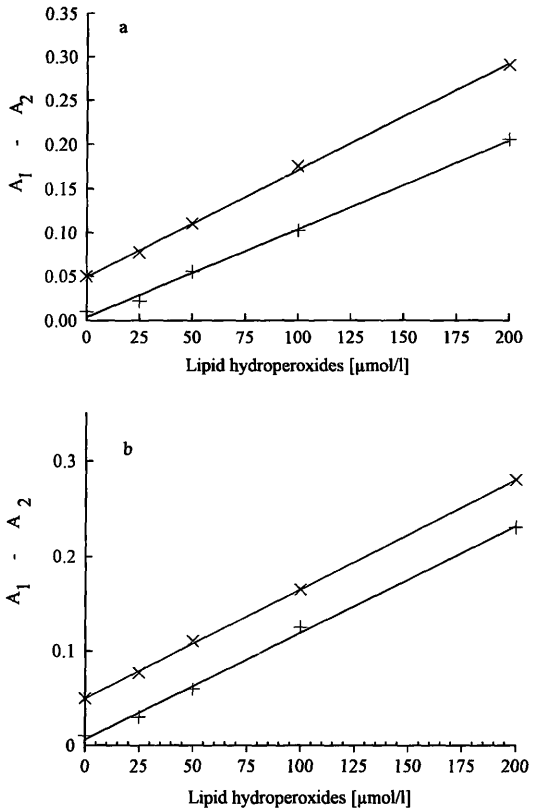

Fig. 2 Plasma lipid hydroperoxides determination. Matrix interference study. Standard solution of *t*-butyl hydroperoxide (+) and standard solution of *t*-butyl added to a constant amount of plasma pool (x); a) aqueous solution; b) albumin solution.
A_1 is the absorbance value at 340 nm after 5 minutes of glutathione peroxidase addition and A_2 the absorbance value at 340 nm after 10 minutes of glutathione reductase addition.

Both sets were analyzed by applying the modified assay. The calibration curves obtained for each set are shown in figure 2. The covariance analysis reveals significant differences at a 95% probability level ($F = 49.17$, $F_{tab} = 7.71$).

In order to ascertain whether the interferences were due to the absence of proteins in the standard solutions, the following assay was carried out: the standard solution of *t*-butyl hydroperoxide 300 µmol/l was prepared from the 60 mmol/l stock solution in a bovine albumin solution 65 g/l in 9 g/l NaCl (17), and this solution was used instead of water to prepare the set of standards. In these conditions when the matrix interference assay was carried out there were no significant differences between the slopes of the curves obtained with and without matrix addition, at a 95% probability level ($F = 4.54$; $F_{tab} = 7.71$). The curves obtained are shown in figure 2. Therefore, bovine albumin (65 g/l in 9 g/l NaCl) was used thereafter as the standard solvent.

Analytical properties

a) Linearity

The range of linearity was determined by duplicate analysis of a set of *t*-butyl hydroperoxide solutions, prepared from a stock solution of 60 mmol/l, and a set of hydrogen peroxide, at the following concentrations: 25, 50, 100, 150, 200, 250, 300 and 350 µmol/l. The results obtained are graphically expressed in figure 3. The linearity was excellent up to 350 µmol/l.

- *t*-butyl hydroperoxide $r = 0.998$; $y = 4.0 \cdot 10^{-4} + 1.0 \cdot 10^{-3}x$
- hydrogen peroxide $r = 0.999$; $y = 4.7 \cdot 10^{-4} + 1.0 \cdot 10^{-3}x$

This value was in the linearity limit, however, because this amount of *t*-butyl hydroperoxide practically exhausts all the NADPH present, and therefore it is advisable to dilute the samples with contents higher than 300 µmol/l.
The manual and automated assays had similar ranges of linearity.

b) Precision

A within-run precision assay was performed by analyzing 10 aliquots of a plasma pool, and 10 aliquots of the sample plasma pool, diluted 1 to 2, in bovine albumin. In the manual assay the means and variation coefficients were 66.13 µmol/l and 7.75%, and 33.31 µmol/l and 11.54%, of the undiluted and diluted plasma pools, respectively. In the within-run assay (automated method) the variation coefficients of 3.34 and 5.02% corresponded to contents of 80.20 µmol/l and 39.54 µmol/l, respectively. To obtain the day-to-day precision, 10 aliquots of a plasma pool were analyzed

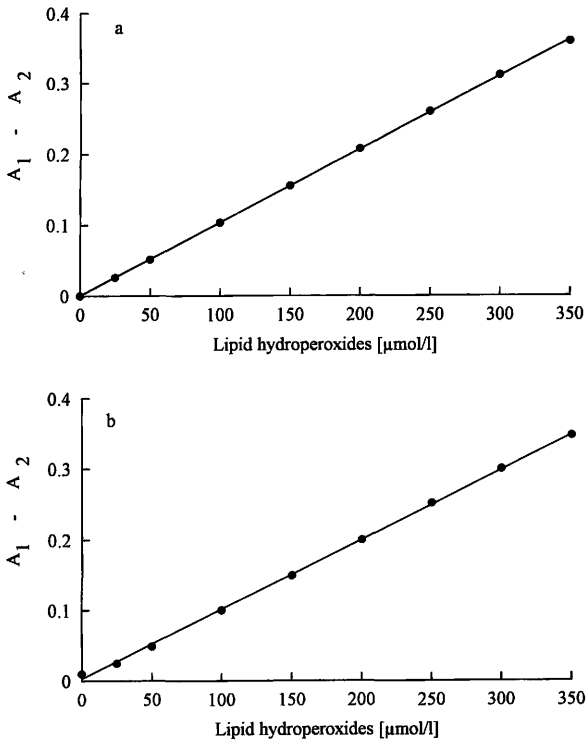

Fig. 3. Plasma lipid hydroperoxides determination. Linearity assay:
a) Butylated hydroxytoluene $y = 4.0 \cdot 10^{-4} + 1.0 \cdot 10^{-3}x$; $r = 0.998$;
b) Hydrogen peroxide $y = 4.7 \cdot 10^{-4} + 1.0 \cdot 10^{-3}x$; $r = 0.999$.
A_1 is the absorbance value at 340 nm after 5 minutes of glutathione peroxidase addition and A_2 the absorbance value at 340 nm after 10 minutes of glutathione reductase addition.

for a period of 10 days (aliquots of the sample were frozen at −20 °C). The values obtained were 69.69 µmol/l and 9.76% and 81.36 µmol/l and 5.8% for the manual and automated assays, respectively.

c) Analytical sensitivity

The blank value, that is, the decrease in absorbance, when it existed, was very low. Therefore, the detection limit could not be calculated and the analytical sensitivity − the change in the signal relative to a change in the concentration of the analyte − was estimated instead. The minimal amount of *t*-butyl hydroperoxide added to a sample that can be differentiated from the sample content was estimated using the following criteria (18):

$$C_m + 3\,s < C_{ma} - 3\,s$$

where C_m was the mean of the sample measurements, C_{ma} was the mean of the added sample measurements, and s was the standard deviation.

The assay was performed by adding small decreasing amounts (25 to 5 µmol/l) of *t*-butyl hydroperoxide solution to a plasma pool and analyzing the plasma pool with and without additions.

Tab. 1 Detection limits[1] of the manual and automated modified methods.

	Manual method		Automated method	
	C_m	3s	C_m	3s
Sample	59.2	12.3	70.1	15.3
	C_{ma}	3s	C_{ma}	3s
Sample + *t*BHT 5 µmol/l	65.0	12.3	76.6	6.6
Sample + *t*BHT 10 µmol/l	73.0	15.3	82.0	6.0
Sample + *t*BHT 15 µmol/l	76.7	12.0	85.1	12.0
Sample + *t*BHT 20 µmol/l	82.2	9.6	89.3	12.0
Sample + *t*BHT 25 µmol/l	86.3	14.1	94.8	9.6

[1] Minimal amount of *t*-butyl hydroperoxide (*t*BHP) added to a sample that can be differentiated from the sample content estimated by using the following criteria $C_m + 3s < C_{ma} - 3s$, C_m being the mean of the sample measurements and C_{ma} the mean of the added sample measurements. Ten aliquots were analyzed in each case and s was the standard deviation.

The results obtained are shown in table 1. The detection limits obtained by applying this criterion were 20 µmol/l and 10 µmol/l for the manual and automated methods, respectively.

d) Accuracy

As there were neither lipid hydroperoxides reference materials nor a reference method to use to evaluate the accuracy of the method, a recovery assay was used for this purpose. Two sets of plasma pool, to one of which 50 µmol/l of *t*-butyl hydroperoxide was added, were analyzed. The recovery values (%) expressed as mean and standard deviation were 98.39 ± 9.88 and 98.70 ± 4.89 for the manual and automated assay, respectively.

Reference interval

Lipid hydroperoxides concentrations were determined in the plasma of 90 blood donors (age range: 18−62 years, 45 male and 45 female) in the blood donors centre of transfusions in Castellón, Spain. The samples of the donors were taken into vacutainer tubes (Vacutainer EDTA · K_3) by the blood transfusion center.

After centrifugation, butylated hydroxytoluene (10 µl of butylated hydroxytoluene, 20 g/l) was added to plasma samples. The procedure described above was applied to the samples in order to determine the lipid hydroperoxides content.

The following lipid hydroperoxides (µmol/l) values were obtained: mean 67.86; standard deviation (SD) 15.00; mean ± 2 SD: 37.86−97.86

Discussion

The coupled enzymatic technique using glutathione reductase described by *Heath & Tappel* (14) is a good candi-

date for routine determination of the degree of peroxidation in the organism. However, this method is susceptible to various kinds of interference that make it unsuitable for plasma lipid hydroperoxides estimation (19).

In applying the *Heath & Tappel* method to plasma samples a difference between the kinetics of reaction of the standards and samples was observed. In the case of the samples, during the second incubation, carried out to achieve the lecture stabilization, the values continued to decrease, and therefore, there is no stop of the reaction. Furthermore, after 15 min in the third incubation, when the second lecture has to be done, the decrease of the absorbance follows.

Kohda et al. (20) explain the different behaviour of standards and samples in terms of the interference caused by plasmatic endogen enzymes involved in the reactions used in the determination, and enzyme inactivation techniques have been applied to correct the deficiencies of the studied method (21). However, we have observed that the differences in behaviour between standards and samples can be suppressed by adding butylated hydroxytoluene, which is able to prevent in vitro peroxidation (22, 23).

On the other hand, it was not considered necessary to carry out the incubation at 33 °C, and the incubation was at room temperature. The temperature of incubation has also been modified by other authors (24, 25).

Matrix interferences were observed when aqueous standards were used. Therefore, it is advisable to prepare the standards in bovine albumin at a concentration of 65 g/l in 9 g/l NaCl.

This new protocol and the automated assay give acceptable within-run precision with a coefficient of variation of 3.34%, as well as acceptable between-run precision (CV 5.80%) at the usual lipid hydroperoxides plasma concentration. In addition, the recovery is very satisfactory (98.7 ± 4.89%). The method is linear for a wide range of contents and sensitive enough to measure the lipid hydroperoxides plasma content.

The automated assay requires only a fraction of the time necessary for the manual method, 30 samples can be analyzed in 20 min, and the precision and sensitivity improve dramatically with respect to the manual procedure. In addition, the automated procedure requires less attention of the analyst and smaller amounts of samples and reagents (1/4 part) than the manual one. On the other hand, in the Cobas FARA instrument the absorption is independent of the final volume, and is only influenced by the accuracy of the sample measure.

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